REMARKS

Applicants respectfully request entry of the amendment and reconsideration of the claims. Claims 16 and 19 have been amended to further clarify the claimed invention. The amendment is supported by the specification, including for example at page 5, lines 27-31 and Fig. 5, and does not introduce any issues of new matter. Applicants submit the amendment places the claims in condition for allowance.

Claims 1-3, 5, 6, 9, 10, and 16-24 are currently pending. The Examiner indicated claims 1-3, 5, 6, 9, 10, 18 and 22 are allowable. Applicants thank the Examiner for allowance of these claims.

Obviousness

Claims 16-17, 19-21, 23, and 24 were rejected under 35 U.S.C. § 103(a) as unpatentable over U.S. Patent No. 6,099,832 (Mickle et al.) in view of WO97/44070 (Shapiro et al.). The Office Action alleges it would have been obvious to substitute the alginate scaffolds disclosed in Shapiro et al. for the collagen scaffolds taught by Mickle et al. Applicants respectfully traverse this rejection.

The Patent Office bears the initial burden of factually supporting any *prima facie* conclusion of obviousness. MPEP § 2142. Three criteria must be established for a *prima facie* case of obviousness. First, there must be some suggestion or motivation, either in the references themselves or in the knowledge generally available to one of ordinary skill in the art, to modify the reference or to combine the teachings. Second, there must be a reasonable expectation of success. Finally, the prior art reference must teach or suggest all the claim limitations. In re Vaeck, 947 F.2d 488 (Fed. Cir. 1991). One or more of the three criteria has not been established.

Claim 16 as amended recites that the cells cultured in the porous polysaccharide matrix form a multicellular aggregate. Neither Mickle et al. or Shapiro et al., alone or in combination, teach or suggest a tissue engineered cardiac biograft comprising a multicellular aggregate of cardiomyocytes. The combination of references cited in the Office Action therefore fails to teach or suggest all of the elements of the claim.

Moreover, one of skill in the art could not predict the outcome of a cardiac biograft comprising an alginate scaffold in view of the collagen-based cardiac biograft disclosed by

Mickle et al. Unlike collagen, alginates do not promote adhesion of cells (see, for example, Rowley et al., 1999, Biomaterials, 20:45-53 (copy enclosed)). The adhesive properties of collagen-based matrices therefore result in a completely different product. One of skill in the art would not have been motivated to combine Mickle et al. and Shapiro et al. as the ECM binding properties of collagen-based biografts and the cell adherence properties of collagen-based biografts are substantially different from those of alginate-based scaffolds.

Accordingly, Applicants assert that the Office Action has failed to establish a *prima facie* case of obviousness and the claims patentably distinguish over the combination of references. Withdrawal of the rejection is respectfully requested.

Written Description

Claims 16-17, 19-21, 23, and 24 were rejected under 35 U.S.C. § 112, first paragraph, as failing to comply with the written description requirement. Without acquiescing the rejection and solely for the purpose of advancing prosecution, the non-adhesive limitation has been removed from claims 16 and 19. Withdrawal of the rejection is respectfully requested.

Summary

In view of the above amendments and remarks, Applicants respectfully request removal of the finality, entry of the amendments, and consideration of the Remarks. If the Examiner believes a telephone conference would advance the prosecution of this application, the Examiner is invited to telephone the undersigned at (612) 332-5300.

Respectfully submitted,

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Biomaterials

Alginate hydrogels as synthetic extracellular matrix materials

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Abstract

Alginate hydrogels are used extensively in cell encapsulation, cell transplantation, and tissue engineering applications. Alginates possess many favorable properties required in biomaterials, but are unable to specifically interact with mammalian cells. We have therefore covalently modified alginate polysaccharides with RGD-containing cell adhesion ligands utilizing aqueous carbodiimide chemistry. The chemistry has been optimized and quantified with reaction efficiencies reaching 80% or greater. The concentration of peptide available for reaction was then varied to create hydrogels with a range of ligand densities. Mouse skeletal myoblasts were cultured on alginate hydrogel surfaces coupled with GRGDY peptides to illustrate achievement of cellular interaction with the otherwise non-adhesive hydrogel substrate. Myoblasts adhere to GRGDY-modified alginate surfaces, proliferate, fuse into multinucleated myofibrils, and express heavy-chain myosin which is a differentiation marker for skeletal muscle. Myoblast adhesion and spreading on these GRGDY-modified hydrogels was inhibited with soluble ligand added to the seeding medium, illustrating the specificity of adhesion to these materials. Alginate may prove to be an ideal material with which to confer specific cellular interactive properties, potentially allowing for the control of long-term gene expression of cells within these matrices.

Keywords: Alginates; Hydrogels; Synthetic ECMs; Tissue engineering; Biomaterials; Skeletal myoblasts

1. Introduction

Hydrogels have an extensive history of use in medicine, pharmacy, and the basic sciences [1]. Various hydrogels, both synthetic and naturally derived, have recently been used as synthetic extracellular matrices (ECMs) for cell immobilization, cell transplantation and tissue engineering [2–5]. Synthetic ECMs replace many functions of the native ECM, organizing cells into a three-dimensional architecture, providing mechanical integrity to the new tissue, and providing a hydrated space for the diffusion of nutrients and metabolites to and from the cell [5–7].

Alginates are naturally derived polysaccharides and have been extensively used as hydrogel synthetic ECMs [8]. Alginates are composed of (1-4)-linked β-D-mannuronic acid (M units) and α-L-guluronic acid (G units) monomers which vary in amount and sequential distribution along the polymer chain depending on the source of the alginate [9]. The alginate molecule is a block copolymer composed of regions of sequential M units (M-blocks), regions of sequential G units (G-blocks), and regions of atactically organized M and G units. Divalent cations like Ca^{2+} cooperatively bind between the G-blocks of adjacent alginate chains, creating ionic interchain bridges which cause gelling of aqueous alginate solutions. It is important to note that ionically cross-linked alginates lose mechanical properties over time in vitro, presumably due to an outward flux of cross-linking ions into the surrounding medium [7]. To address this concern, stable covalent cross-links may be introduced into alginate hydrogels using bifunctional cross-linkers [10], allowing for greater control over the mechanical and swelling properties of the gels. Alginate/cell suspensions may also be gelled in situ,

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providing a means for cell transplantation with minimally invasive surgical procedures [4]. However, mammalian cells are unable to specifically interact with alginate polysaccharides or their hydrogels either naturally or through serum proteins, as alginate hydrogels promote minimal protein adsorption [11].

Cell anchorage is a strict requirement for survival of most cell types, and it orchestrates critical roles in many cellular functions including migration, proliferation, differentiation, and apoptosis [12, 13]. Cell interaction with biomaterials is mediated through transmembrane receptors which recognize adhesion molecules at materials surface [14]. Many polymer systems promote the physical adsorption of cell adhesion molecules from surrounding fluids due to a thermodynamic driving force, and proteins in solution are extensively deposited onto these hydrophobic surfaces [15]. However, alginate and other hydrogels are able to discourage protein adsorption due to the hydrophilic nature of the polymers [11]. Since cell adhesion is a strict requirement for survival, most cells must be cultured in alginate hydrogels as multicellular aggregates [16].

We are covalently modifying alginate hydrogels with cell adhesion ligands to promote cell anchorage and interaction with these materials. The carboxylic acid functional groups along the alginate polymer chain offer the potential for covalent modification with specific cell-adhesion molecules such as RGD-containing peptides. In this paper we outline the chemistry used for the covalent modification of alginate with RGD-containing peptides, and illustrate increased cellular interaction with these materials using mouse skeletal myoblasts as the cell system. Alginate is an ideal polymer to engineer in specific cellular interactive characteristics, as the lack of interaction between mammalian cells and the native alginate hydrogels allows for alginate to act as a 'blank slate'. The resulting polymers may have a high signal to noise ratio, which will be ideal for studying cellular interactions with specific ligands. In addition, alginate polymers meet many requirements for an ideal matrix material, in that they are well characterized, are amenable to sterilization and storage, and may be chemically modified with simple chemistries [17].

2. Materials and methods

2.1. Materials

ProNova MVG, a high G content alginate, was purchased from ProNova Biopolymers (Norway). The pentapeptide Glycine–Arginine–Glycine–Aspartic Acid–Tyrosine, abbreviated as GRGDY which are the single letter amino acid abbreviations, was produced by the Protein and Carbohydrate Structure Core facilities at the University of Michigan. Custom iodination through the carboxy-terminal tyrosine of the GRGDY peptide was performed by Biomedical Technologies Inc. (Stoughton, MA) to yield the ^{125}I-labeled GRGDY (^{125}I-GRGDY) with a specific activity of 0.74 μCi μg^{-1} peptide for peptide incorporation studies. All other chemicals were purchased from Sigma (St. Louis, MO) unless otherwise stated.

2.2. Chemistry

Alginate was chemically modified utilizing aqueous carbodiimide chemistry. 1-ethyl-(dimethylaminopropyl) carbodiimide (EDC), a water-soluble carbodiimide [18], was used to form amide linkages between amine containing molecules and the carboxylate moieties on the alginate polymer backbone. The co-reactant *N*-hydroxysulfosuccinimide (sulfo-NHS, Pierce, Rockford, IL) stabilizes the reactive EDC-intermediate against a competing hydrolysis reaction, raising the efficiency of amide bond formation [18]. The GRGDY pentapeptide was conjugated to the alginate polymer backbone through the terminal amine of the peptide (Fig. 1). Reactions were carried out in a 0.1 M MES buffer and the chemistry was optimized with regard to the carbodiimde:uronic acid ratio, pH, and NaCl molarity. Peptide incorporation was quantified using radiolabeled ^{125}I-GRGDY as a tracer molecule.

2.3. Method 1—solution modification

Alginate was modified with the GRGDY peptide in solution to create a homogeneously modified material.

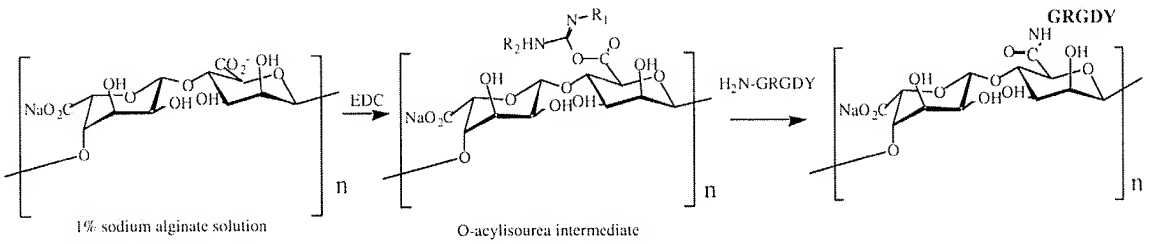

Fig. 1. Reaction scheme of peptide coupling to alginate molecules. Amide bond formation is mediated by the carbodiimide through the carboxyl group of the alginate and the N-terminal amine of the GRGDY pentapeptide.

The chemistry was optimized for a peptide density of 1 mg GRGDY per gram alginate as it is 2.5 orders of magnitude greater than the minimal RGD ligand spacing determined necessary for cell attachment [19] when extrapolated to three-dimensional space (calculations based on a body centered cubic unit cell). Alginate chemistry was performed in 1% (v/v) alginate solutions in 0.1 M MES buffer at varying pH (6.0–7.5) and NaCl concentrations (0.0–0.7 M) for 12 or 20 h. Sulfo-NHS was disolved in the alginate solution at a ratio of 1 : 2 to EDC, and EDC was next added as a percentage of uronic acids available for reaction (0–50%). The GRGDY peptide was added after 5 min with ^{125}I-GRGDY as a tracer molecule (activities of 2–5 μCi per reaction). The alginate product was purified by dialysis (3500 MWCO) against ddH_2O for four days and lyophilized until dried. The resultant solid was weighed and dissolved in ddH_2O to obtain a 0.5% (w/v) solution, of which the activity of 0.5 ml samples (three per condition) were counted with a Packard-Bell Gamma Counter. The activity in counts per minute were compared to the initial reaction solution activities, and reaction efficiencies were calculated taking into account the ^{125}I decay. A range of ligand densities in the bulk was produced by using optimized chemistry (see Results) and changing the GRGDY concentrations in the reactions.

2.4. Method 2—hydrogel modification

In the second method, pre-formed hydrogels were modified with the GRGDY peptide using similar chemistries. Calcium cross-linked alginate hydrogels were prepared from 2% (v/v) alginate solutions in ddH_2O containing 0.2% (w/v) $Na(PO_4)_6$ (Alfa, Ward Hill, MA). Calcium sulfate was added to alginate in 50 ml centrifuge tubes as a water-based slurry at 0.41 g $CaSO_4$/ml ddH_2O, with 0.2 ml of the slurry added for every 5 ml of the 2% alginate solution to be gelled. The gelling solution was shaken rapidly and cast between parallel glass plates with 2 mm spacers to prepare gel films. Hydrogel disks were punched out of the film with a hole punch (McMaster-Carr, Chicago, IL) for modification of the hydrogel. The hydrogel disks were derivatized with RGD using unbuffered EDC chemistry in ddH_2O with sulfo-NHS as the co-reactant. Sulfo-NHS and EDC were added to 40 ml ddH_2O at the same ratios as modification Method 1, followed by addition of the GRGDY peptide. Method 2 reactions were performed in 50 ml centrifuge tubes on 10–12 hydrogel disks at a time for 20 h. Surface densities of GRGDY were estimated with this method assuming a 50 nm penetration of reactants, since uronic acid available for reaction greatly outnumbered the molar quantities of reactive species. Peptide surface densities were quantified with the ^{125}I-GRGDY tracer molecule as described above. All cell experiments were performed on Method 2 modified disks.

2.5. Cell studies

C2C12 skeletal myoblasts (ATCC), passage number 2–7, were used to study cell attachment, proliferation, and differentiation on alginate hydrogel surfaces. Alginate hydrogel disks modified to contain RGD surface densities of approximately 1 nmol cm^{-2} or unmodified (control) alginate disks were used for these experiments. The cell culture medium consisted of high glucose DMEM with 10% FBS and penicillin–streptomycin at 100 units per ml (all from Gibco BRL, Gaithersburg, MD). Myoblasts were seeded onto these disks in 12 well tissue culture plates at a density of 25 000 cells cm^2. All plates were rinsed with fresh medium at 4 h to remove unattached cells from the hydrogel surfaces, and cell counts were performed at 4, 24, 72, and 120 h. For cell counts, alginate disks were added to 1 ml of trypsin in 15 ml centrifuge tubes and held at 37°C for 5 min to disrupt cell–cell and cell–matrix interactions, and then 10 ml of 2% (w/v) sodium citrate was added to each tube to dissolve the alginate disks. After alginate dissolution (20–30 min), cells were pelleted by centrifugation, resuspended following removal of the supernatant, and cell counts were performed in triplicate using a Coulter Counter. Ligand competition experiments were performed to demonstrate the specificity of attachment of cells to alginate hydrogel surfaces. Soluble GRGDY was added to the cell seeding medium at concentrations of 0.01, 0.1, and 1.0 mM, and cell counts were performed at 4 h after cell seeding to determine attachment efficiencies in these mediums.

Alginate disk cultures were rinsed with PBS and fixed with 4% formaldehyde for five minutes to prepare for immunostaining. Between all steps the cultures were rinsed thoroughly with a tris-buffered saline (TBS) solution containing 0.1% Triton. Cells were permeabilized with 0.5% Triton X-100 solution in TBS for 10 min, and primary antibody F1.652 for myosin heavy chain[20] (DSHB, Iowa City, IA) or a DAPI nuclear stain was added to the cultures for 1.5 h and 1 min, respectively. The myosin-staining cultures were then incubated with a rhodamine conjugated secondary antibody (goat anti-mouse) (Sigma, St. Louis, MO) for 45 min, followed by DAPI to counterstain for the nucleus. Alginate disks were mounted on glass slides with Crystal/Mount mounting medium (Biomeda Corp, Foster City, CA) and a coverslip, and were then sealed with a mixture of vaseline and wax. The cells were visualized with a Nikon Eclipse E800 fluorescent microscope, and images were digitally obtained in Photoshop or taken with a Nikon camera attachment.

3. Results

3.1. Chemistry

To optimize peptide incorporation for bulk modified alginate, the minimum amount of EDC necessary for

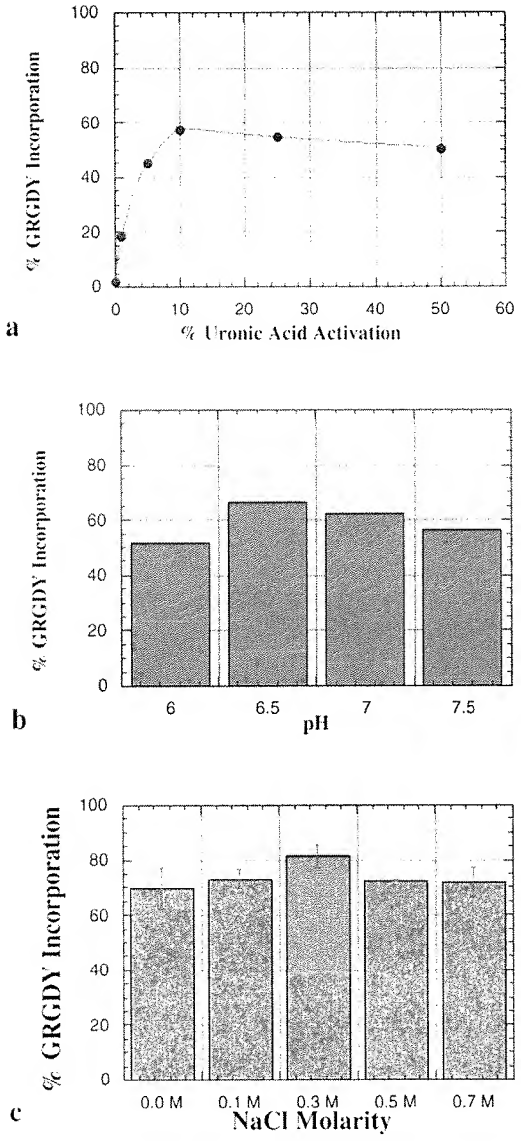

Fig. 2. Quantification of peptide incorporation using I^{125}-GRGDY as a tracer molecule: (a) the theoretical uronic acid activation was varied by altering the concentration of EDC added to the reaction medium (pH 7.0, 0.5 M NaCl, reaction time 12 h, standard deviation is within the size of the symbols in the graphs); (b) the pH was next varied (5% uronic acid equivalents of EDC, 0.5 M NaCl, reaction time of 20 h), and (c) then the NaCl concentration was altered (5% uronic acid equivalents of EDC, pH 6.5, reaction time of 20 h). All values represent the mean of at least three samples (each counted twice) taken from the same reaction condition, except for the final optimization reaction (c) where activities were the mean of at least three samples (each counted twice) taken from three different reactions of the same condition.

^{125}I-GRGDY incorporation was first determined. The EDC content was varied in this first experiment as a percentage of the carboxylic acid groups available for reaction on the alginate. GRGDY incorporation increased with increasing carbodiimide and reached a maximum at 10% theoretical activation of uronic acids by the carbodiimide (Fig. 2a). The 5% uronic acid activation chemistry was next used for the optimization of ^{125}I-GRGDY incorporation for pH and NaCl content to minimize side reactions associated with carbodiimide chemistry (see discussion). GRGDY incorporation was greatest at pH 6.5 with a reaction efficiency of approximately 78%. The NaCl molarity of the 0.1 M MES buffer was then varied from 0.0 to 0.7 M with optimal incorporation at 0.3 M NaCl in the reaction medium (Fig. 2c). Control reactions without EDC were run with every experiment and less than 1% of ^{125}I-GRGDY remained after dialysis, suggesting that the incorporation values are not a result of nonspecific interactions between the polysaccharide and the peptide adhesion ligand.

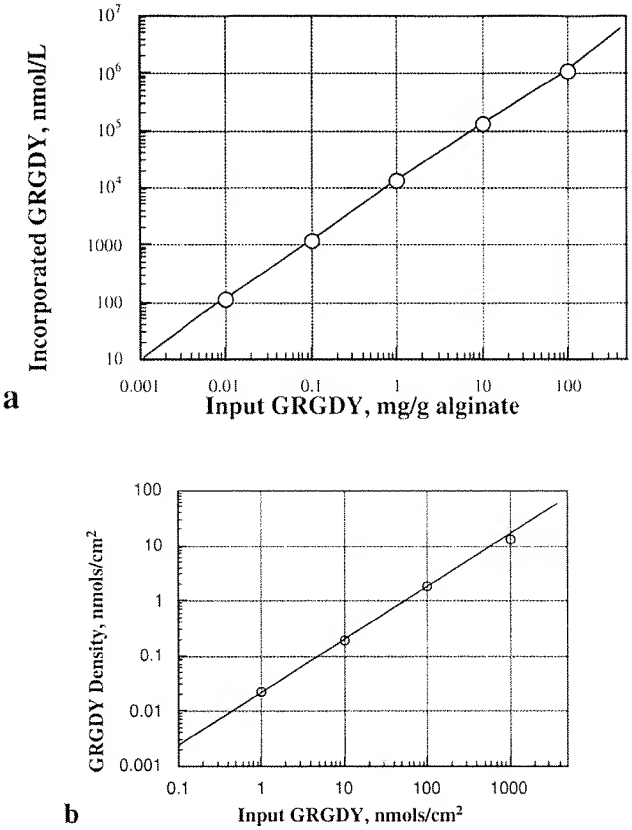

Fig. 3. Coupled GRGDY peptide as a function of the amount of input peptide in bulk-modified alginate (a), and surface-modified alginate (b). Values in (a) represent the mean of at least three samples (each counted twice) taken from a single reaction condition (standard deviation is within the size of the symbols in the graph), and values in (b) represent the mean of at least four samples at each condition (each counted twice, standard deviation is within the size of the symbols in the graph).

A number of past studies indicate that a variety of cellular functions are controlled by the adhesion ligand density presented to cells [20–23]. Control of cell adhesion ligand densities over several orders of magnitude will thus likely allow for future control of cellular function. The concentration of peptide available for reaction was varied over a five-order of magnitude range in the bulk (Method 1 modification), and over a four-order of

magnitude range for surface concentrations (Method 2 modification). In both cases, a linear relationship between GRGDY incorporation and peptide available for reaction was found (Fig. 3a and b).

3.2. Cell studies

Cell culture experiments with C2C12 mouse skeletal myoblasts were utilized to illustrate the increased biological interaction of mammalian cells with the covalently modified alginates discussed above. Myoblasts seeded onto GRGDY-coupled alginate hydrogels (GRDGY density $\approx 1\ \text{nmol cm}^{-2}$) attached and began spreading by 4 h (Fig. 4a), while no myoblast adhesion was observed on control alginate surfaces (Fig. 4b). Myoblast spreading was greatly enhanced at 24 h (Fig. 4c), and cell number increased by day 3 (Fig. 4d). A range of negative controls were run to verify that the coupled GRGDY peptide was responsible for the myoblast adhesion to the alginate hydrogel surfaces, and that adhesion was not a result of nonspecific adsorption or covalent modification of the surface due to reaction between nascent activated carboxyls and the proteins in the serum containing medium. Specifically, no cell adhesion was observed when myoblasts were seeded onto hydrogel surfaces reacted with EDC and sulfo-NHS without GRGDY, hydrogels reacted with sulfo-NHS and GRGDY, or hydrogels reacted with GRGDY without the carbodiimide. The specificity of cellular adhesion to the GRGDY-modified surfaces was further illustrated by adding soluble GRGDY to the cell seeding medium to compete for binding sites with the GRGDY-coupled to the alginate. Cell attachment, but no cell spreading, was observed in cell-seeding medium (DMEM +10% FBS) with soluble GRGDY concentrations of 0.01, 0.1 and 1.0 mM (Fig. 5a). Quantification of the number of cells attached to the GRGDY-modified surfaces indicated a 9-fold decrease in cell adhesion with soluble GRGDY present in the seeding medium (Fig. 5b).

Myoblast proliferation on hydrogel surfaces reacted with the GRGDY peptide was next quantified (Fig. 6). Myoblast number increased only slightly in the first 24 h after cell seeding. The extent of cell spreading however was greatly increased at this time (Fig. 4c).

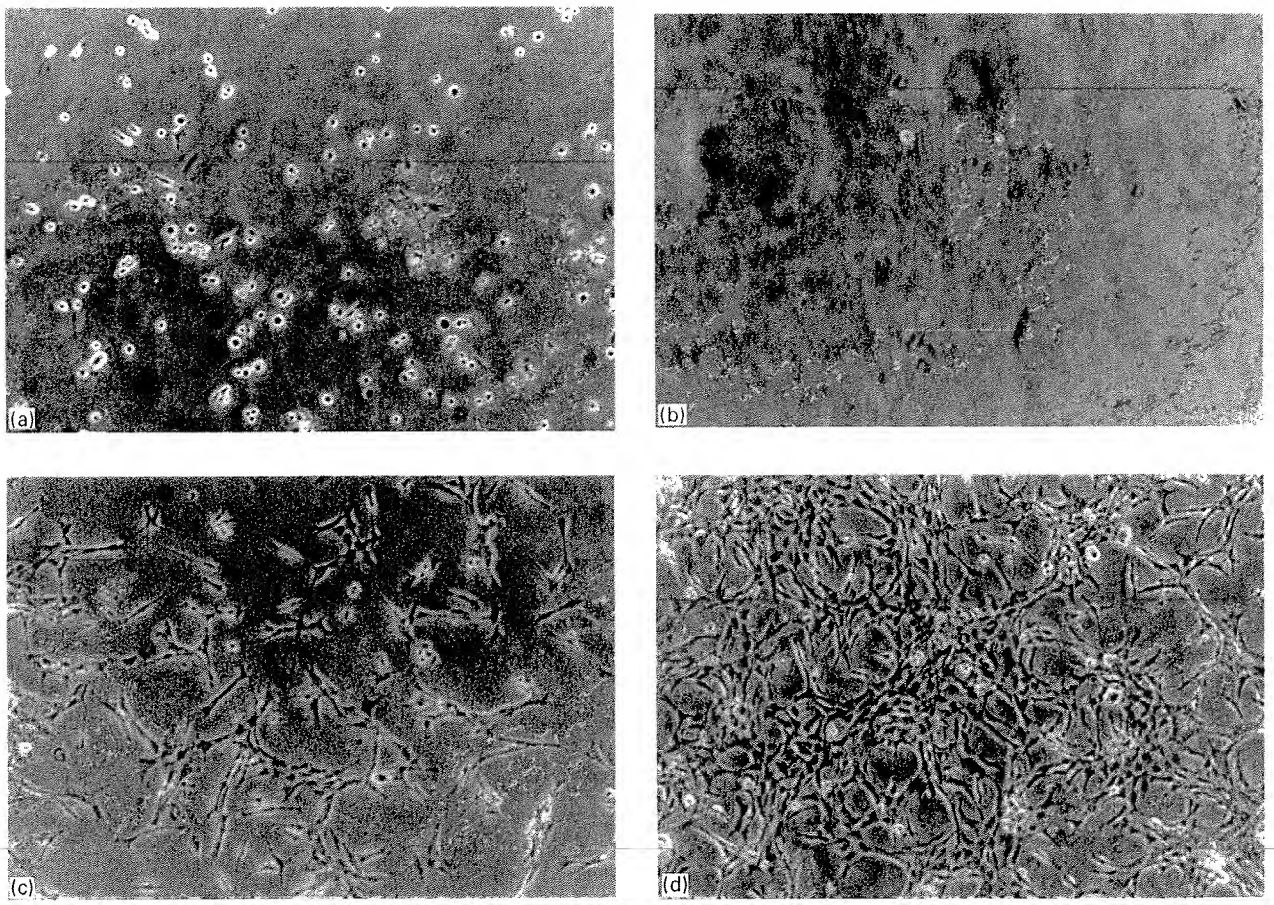

Fig. 4. Photomicrographs of myoblasts adherent to alginate hydrogel surfaces after medium changing at 4 h post-seeding on GRGDY-modified surfaces (a), and control alginate surfaces (b).The myoblasts on GRGDY-modified alginate spread extensively after 24 h of culture (c), and proliferated greatly between days one and three (d).

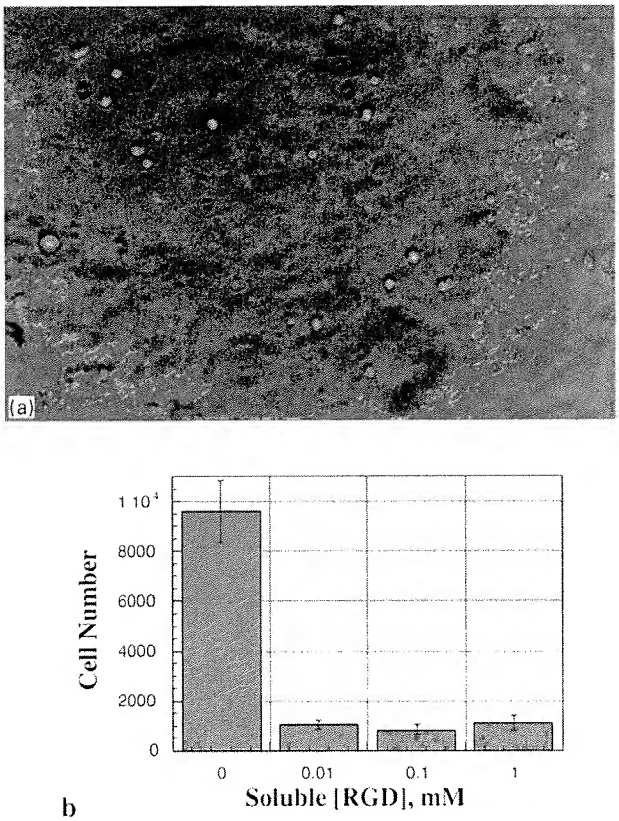

Fig. 5. (a) Photomicrograph of cells adherent to GRGDY-modified alginate in medium (DMEM + 10% FBS) with soluble GRGDY (0.1 mM) after 4 h. Note the decreased attachment efficiency and cell spreading compared to Fig. 4a. (b) The quantification of the number of cells attached to the GRGDY-modified surfaces after 4 h with soluble GRGDY present in the seeding medium.

Myoblasts proliferated greatly between days one and three (Figs. 4d and 6), and the number of cells per gel reached a maximum at 72 h. The cell number per gel decreased after day three, but this does not appear to be due to cell death or apoptosis. The decrease in cell number is instead a result of the fusion of myoblasts into multinucleated myofibrils, which is one step in the pathway of skeletal muscle differentiation [24].

Fluorescent staining of myoblasts in day three cultures on the GRGDY-coupled hydrogel surfaces illustrates the multinucleated nature of cells at this point in time (Fig. 7a). The differentiated characteristics of the alginate-myoblast cultures were further studied by staining the myoblasts for heavy-chain myosin [20]. Myoblasts adherent to the GRGDY-coupled alginate hydrogels stained positively for this marker, confirming the differentiated gene expression of the myoblasts on the surface-modified alginate gels (Fig. 7b). Control cultures of nonconfluent myoblasts (negative control) on tissue culture polystyrene (TCPS) did not stain for myosin heavy chain (not shown). The uniformity of staining on GRGDY-hydrogel surfaces was greater than myoblasts

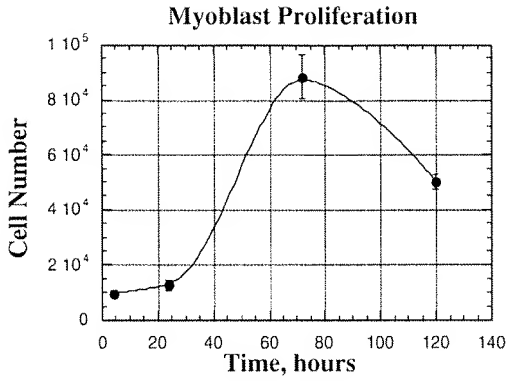

Fig. 6. Quantification of myoblast proliferation on hydrogel surfaces reacted with the GRGDY peptide. Cell counts were taken in triplicate from at least three different hydrogel cultures at each time point.

cultured on TCPS for five days post-confluence (standard positive control [24]). On the GRGDY-hydrogel surfaces virtually all myoblasts stain positively for the heavy-chain myosin, whereas only fully formed myofibrils in the overgrown TCPS cultures stained positively for the marker, and these myofibrils were surrounded by a dense culture of non-fused and non-staining myoblasts (not shown).

4. Discussion

Aqueous carbodiimide chemistry was utilized to covalently couple the cell adhesion peptide GRGDY to alginate polymers in bulk as well as to alginate hydrogel surfaces. This chemistry successfully initiates biological interactions between alginate polymers and mouse skeletal myoblasts, as illustrated by culturing cells on alginate hydrogel surfaces modified with the GRGDY adhesion ligand. This work is the first step in creating a model synthetic extracellular matrix, using alginate as the base material, with which ligand presentation may be varied in a highly controlled manner.

4.1. Optimized chemistry

The amount of EDC present in the reaction had a dramatic effect on peptide incorporation efficiency (Fig. 2a). A common side reaction occurring when EDC is used in large amounts is the internal rearrangement of the O-acylurea activated ester of the EDC to a *N*-acylurea which becomes stably incorporated onto the substrate, in this case the alginate polymer backbone [25, 26]. The amount of *N*-acylurea covalently grafted onto the alginate increases with increasing EDC concentrations when added to aqueous alginate solutions [10], and will likely affect the ionic gelling capacity of the polymer. The 5% uronic acid activation condition offered good

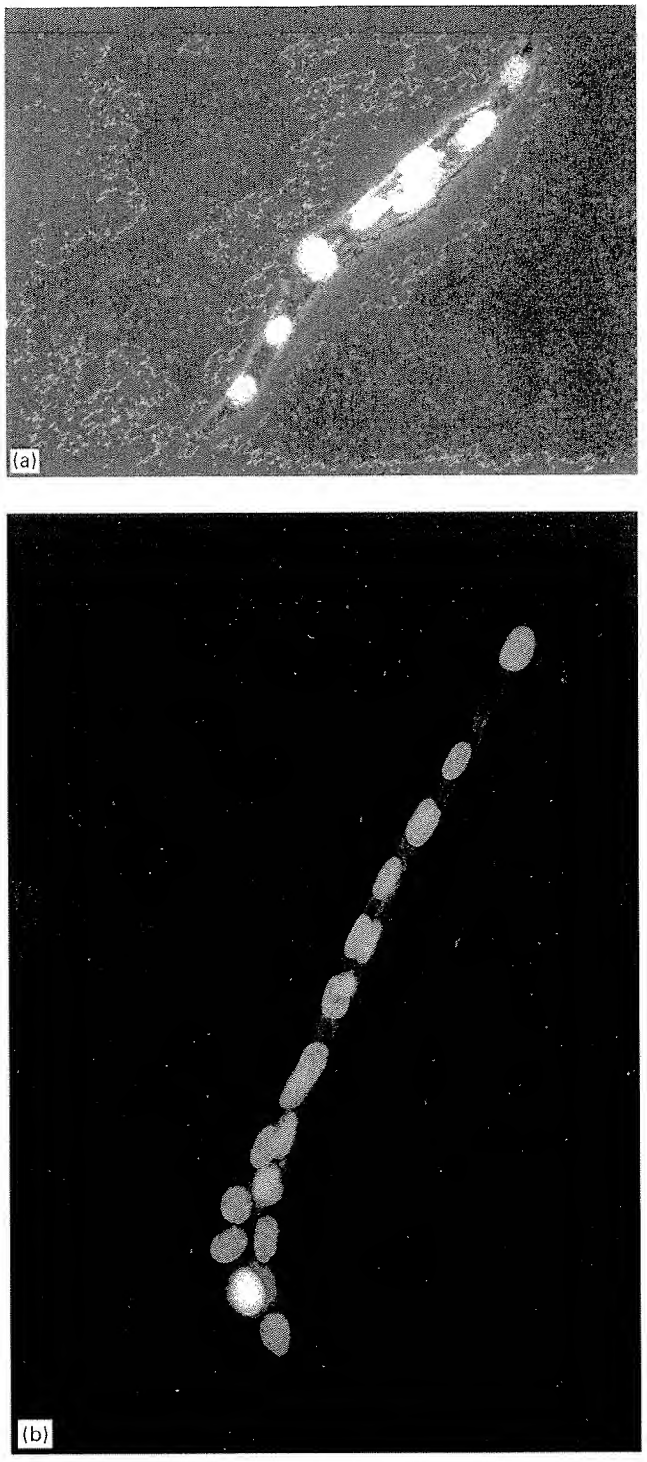

Fig. 7. Photomicrographs of myoblasts cultured on GRGDY-modified alginate surfaces at day three, in which cells were stained with DAPI to highlight nuclei (a), or for myosin heavy chain and DAPI (b).

reaction efficiencies and minimized the extent of the *N*-acylurea rearrangement reaction, and was therefore chosen to optimize the GRGDY peptide incorporation.

The pH and the NaCl concentration of the reaction medium have been reported to affect EDC mediated cross-linking of alginate with diamines [10]. EDC is most reactive at acidic pH (i.e. pH 4.5 [18]). However, a more neutral pH of 6.5 was necessary for optimal peptide incorporation in this system (Fig. 2b). This is presumably due to the p*K*a of the α-amine on the N-terminus of the peptide. The p*K*a of α-amines of amino acids is 7.2–7.4 [18]. The optimal pH of 6.5 found here likely offers a balance in reactivities between the carbodiimide and the α-amine. The NaCl of the reaction medium has a minimal effect on reaction efficiencies in this system (Fig. 2c).

Many researchers have demonstrated that the density of adhesion ligands, in the form of ECM molecules or as synthetic oligopeptide ligands, support a range of cellular responses [20–23]. We have therefore quantified adhesion ligand density in this hydrogel system over physiologically relevant ranges in both two- and three-dimensions (Fig. 3a and b). The minimal ligand spacing for cell adhesion in two-dimensional cultures was determined to be 440 nm [20]. This was extrapolated to three dimensions and correlates to a ligand density of approximately 34 nmol ligand l^{-1}. We have quantified our bulk modified alginate to five orders of magnitude above this density. Similarly, we have characterized surface densities of the GRGDY peptide over four orders of magnitude ranging from 10 nmol cm^{-2} to 10 pmol cm^{-2} (Fig. 3b). The lower limit of characterized densities in both two- and three-dimensional cases was limited by the activity of the ^{125}I-GRGDY tracer molecule in the radioactive experiments. Reaction efficiencies were very similar over all peptide densities, and lower ligand densities may likely be attained simply by limiting the peptide concentration in the reaction.

4.2. Cellular response to GRGDY-derivatized hydrogel surfaces

Ionically cross-linked alginate hydrogel surfaces were covalently modified with the GRGDY peptide to illustrate that incorporation of specific ligands can induce specific cellular interactions with alginate. Skeletal myoblasts seeded onto the surface modified alginate attach and begin spreading by 4 h after seeding (Fig. 4a). Myoblast adhesion to the hydrogel surfaces is through specific interactions with the GRGDY ligand, as is shown by the limited cell adhesion and inhibition of cell spreading on GRGDY-modified surfaces with soluble ligand in the seeding medium (Fig. 5a). Matrix effects on myoblast adhesion and mobility has been well studied [27, 28], and have been attributed to RGD-containing cell adhesion molecules (i.e. fibronectin, vitronectin) and their respective integrins. It is expected that myoblast adhesion to the GRGDY-modified alginate surfaces is directly mediated by similar receptors.

Myoblasts also proliferate, fuse into multinucleated myofibrils, and express a differentiated phenotype on

GRGDY-modified alginate cultures. The differentiation of myoblasts into myofibrils is common in cells cultured on TCPS for multiple days past confluence [24]. However, the myoblasts in this system consistently exhibit membrane fusion and expression of myosin heavy chain in subconfluent cultures within 3 days time (Fig. 7a and b). The early differentiation of myoblasts reported here may be due to the compliant nature of the alginate substratum, as researchers have shown that compliant substratums induce differentiated phenotypes in some cell systems [29, 30].

4.3. Alginate as a biomaterial for tissue engineering

The alginate system reported here provides an attractive model system to study highly specific cell–ligand interactions due to the low protein adsorption of the anionic polysaccharide. The attachment of oligopeptide adhesion ligands has been an established method of inducing cell interaction with materials through integrin-mediated interactions [31]. Peptide ligands have been attached to a variety of materials such as glycophase glass [32], PEG-AA semi-IPNs [33], PVA [34], polyurethane [35], agarose [36], and a photopolymerizable hydrogel system [37]. In contrast to many previously described systems, these alginate hydrogels provide a practical system to translate mechanistic cell–material interaction studies into clinically relevant biomaterials. Alginate is currently utilized in a number of trials to transplant multiple cell types [3, 4] to treat biochemical or structural deficiencies, and alginate matrices optimized for particular cell interactions may be readily used in all of these applications. Few previous model systems possess this ability to be directly used in in vivo situations.

5. Conclusions

Aqueous carbodiimide chemistry has been utilized to covalently graft an RGD-containing cell adhesion peptide to alginate molecules. Mouse skeletal myoblasts adhere, proliferate and express a differentiated phenotype on these materials. This work is the first step in the development of a model synthetic extracellular matrix in which ligand type and density may be readily varied. In addition, we have developed cross-linking chemistries for alginate hydrogels to control the mechanical properties of this hydrogel system [10]. It may therefore be possible to control both the ligand presentation and alter how the matrix responds to cellular interactions by resisting cell tractional forces which follow initial adhesion events. Alginate matrices optimized for particular cellular functions (e.g. migration, differentiation) may then be utilized as cell transplantation matrices to control tissue formation in vivo.

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